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Targeted Lipidomic Analysis in Human Milk Using LC-MS/MS Triple Quadrupole

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Introduction

Human milk contains a highly complex lipidome that is essential for infant nutrition, immune development, and gastrointestinal maturation. Comprehensive lipid analysis is analytically challenging due to wide dynamic ranges, extensive isomerism, and pronounced matrix effects.

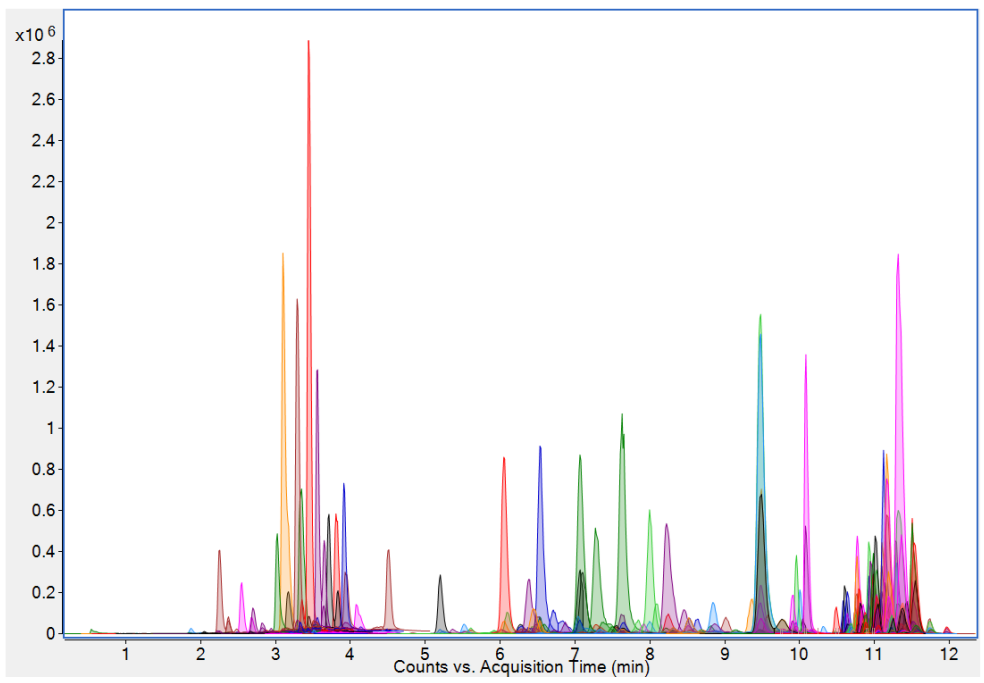
To address these challenges, a high-throughput targeted lipidomics workflow was developed using triple quadrupole LC–MS/MS for robust, sensitive, and quantitative analysis of human milk lipids. Human milk samples were extracted using a 1:1 (v/v) butanol–methanol solvent mix to ensure efficient recovery across diverse lipid classes. Analyses were performed on an Agilent 1290 Infinity III UHPLC coupled to an Agilent 6495D triple quadrupole mass spectrometer operating in both positive and negative electrospray ionization modes. An expanded dynamic MRM panel comprising more than 1,200 lipid targets across over 50 lipid subclasses was employed.

Quantitation was performed using a panel of isotope-labeled internal standards distributing across the retention time range and representative of major lipid classes, with matrix-matched calibration curves prepared pre and post-extraction to correct the extraction loss and matrix effects.

The optimized workflow enabled detection and confirmation of over 800 lipid species in human milk within a 16-minute LC run. Robust and reproducible coverage was achieved across lipid families, including ceramides, cholesteryl esters, diacylglycerols, dehydrocholesteryl esters, free cholesterol, free fatty acids, glycerophospholipids, and triacylglycerols. Spike-in experiments demonstrated strong quantitative performance, with great accuracy, reproducibility and linear calibration responses.

Limits of quantitation as low as ~0.002 (or 0.01 and 0.02) µg/mL were achieved for selected analytes, enabling measurement of low-abundance signaling lipids.

Profile of Lipids in Human Milk



Experimental

Agilent 1290 Infinity III LC with 6495D Triple Quadrupole LC/MS System.

Chromatographic Conditions*

UHPLC: Agilent 1290 Infinity III LC **Column**: Agilent ZORBAX RRHD Eclipse Plus C18, 1.8 µm, 2.1 x 100 mm, pn: 959758-902 **Column oven temperature**: 45 **± 2°C** **Injection volume**: 3 µL **Autosampler**: 20 ± 2°C **Flow rate**: 0.40 mL/min
Mobile Phase A: 10 mM ammonium formate, 5 µM Agilent deactivator additive in 5:3:2 water : acetonitrile : isopropanol Mobile Phase B: 10 mM ammonium formate in 1:9:90 water : acetonitrile : isopropanol

*Huynh, K, et al. A Comprehensive, Curated, High-Throughput Method for the Detailed Analysis of the Plasma Lipidome. Agilent Application Note 5994-3747EN, 2021

MS Conditions-Agilent 6495D Triple-Quadrupole

Parameters	
MS acquisition	Dynamic MRM
Ion source	Agilent Jet Stream electrospray ionization (AJS ESI positive/negative)
Drying gas temperature	150 °C
Drying gas flow	17 L/min
Nebulizer	20 psi
Sheath gas heater	200 °C
Sheath gas flow	10 L/min
Capillary	3500 V ESI+ / 3000 V ESI-
Nozzle voltage	1000 V ESI+ / 1500 V ESI-
iFunnel mode	Standard

The authors declare no competing financial interest

Experimental

Sample Preparation

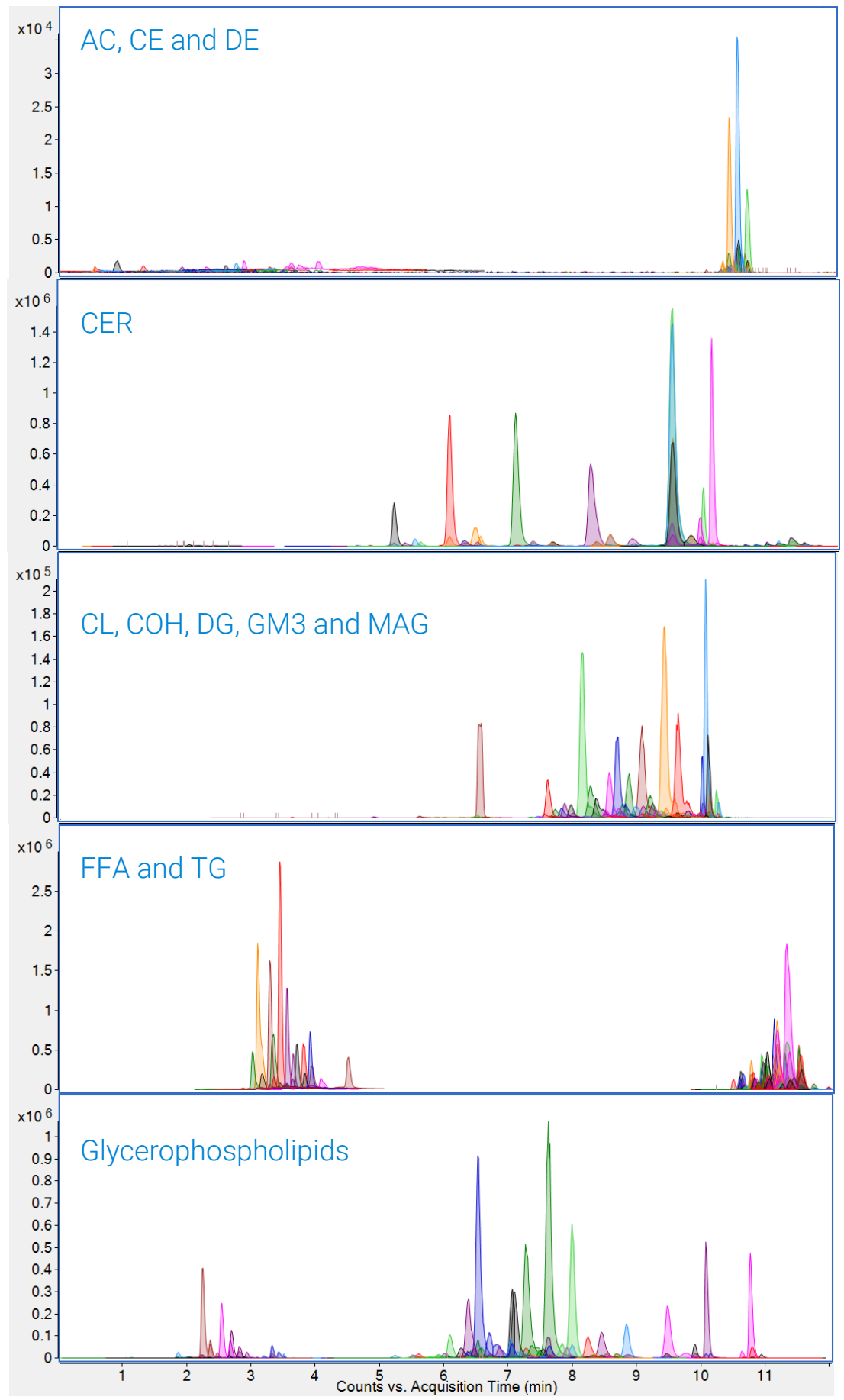
- ✓ Pipet 100 µL human milk into a 2mL centrifuge tube
- ✓ Add internal standard mix
- ✓ Vortex, sonicate
- ✓ Extraction
  - Add 1:1 v:v methanol : butanol to total volume 1mL
  - Vortex and shake for 5 min
  - Centrifuge and collect the supernatant
  - Ready for LCMS injection

Lipids Panel (52 Sub-Classes)

Lipid Class	Lipid Subclass	Full Name
AC	AC	Acylcarnitine
AC	AC-OH	Hydroxylated acylcarnitine
BA	BA	Bile acid
CE	CE	Cholesteryl ester
CE	dimethyl-CE	Dimethyl-cholesteryl ester
CE	methyl-CE	Methyl-cholesteryl ester
Cer	Cer(d)	Ceramide
Cer	Cer(m); dhCER (m)	Deoxyceramide; Dihydrodeoxyceramide
Cer	Cer1P	Ceramide-1-phosphate
Cer	dhCer	Dihydroceramide
Cer	dhCer1P	Dihydroceramide-1-phosphate
Cer	dhHex2Cer	Dihydrodihexosylceramide
Cer	dhHexCer	Dihydromonohexosylceramide
Cer	dhS1P and dhSph	Dihydrosphingosine-1-phosphate
Cer	Hex2Cer	Dihexosylceramide
Cer	Hex3Cer	Trihexosylcermide
Cer	HexCer	Monohexosylceramide
Cer	S1P	Sphingosine-1-phosphate
Cer	SHexCer	Sulfatide
Cer	SM	Sphingomyelin
Cer	Sph	Sphingosine
CL	CL	Cardiolipin
COH	COH	Free Cholesterol
DE	DE	Dehydrocholesterol ester
DE	methyl-DE	Methyl-dehydrocholesteryl ester
DG	DG	Diacylglycerol
FFA	FFA	Free fatty acid
Glycerophospholipids	LPC	Lysophosphatidylcholine
Glycerophospholipids	LPC(O)	Lysoalkylphosphatidylcholine (lysoplatelet activating factor)
Glycerophospholipids	LPC(P)	Lysoalkenylphosphatidylcholine (plasmalogen)
Glycerophospholipids	LPE	Lysophosphatidylethanolamine
Glycerophospholipids	LPE(P)	Lysoalkenylphosphatidylethanolamine (plasmalogen)
Glycerophospholipids	LPG	Lysophosphatidylglycerol
Glycerophospholipids	LPI	Lysophosphatidylinositol
Glycerophospholipids	LPS	Lysophosphatidylserine
Glycerophospholipids	PA	Phosphatidic acid
Glycerophospholipids	PC	Phosphatidylcholine
Glycerophospholipids	PC(O)	Alkylphosphatidylcholine
Glycerophospholipids	PC(P)	Alkenylphosphatidylcholine (plasmalogen)
Glycerophospholipids	PE	Phosphatidylethanolamine
Glycerophospholipids	PE(O)	Alkylphosphatidylethanolamine
Glycerophospholipids	PE(P)	Alkenylphosphatidylethanolamine (plasmalogen)
Glycerophospholipids	PG	Phosphatidylglycerol
Glycerophospholipids	PI	Phosphatidylinositol
Glycerophospholipids	PIP1	Phosphatidylinositol monophosphate
Glycerophospholipids	PS	Phosphatidylserine
GM3	GM3	GM3 ganglioside
MAG	MAG	Monoacylglycerols
OxSpecies	OxSpecies	Oxidised lipids
TG	TG [NL]	Triacylglycerol
TG	TG(O) [NL]	Alkyldiacylglycerol

Results and Discussion

Elution Profile of Lipids Present in Human Milk



Method Validation Procedure

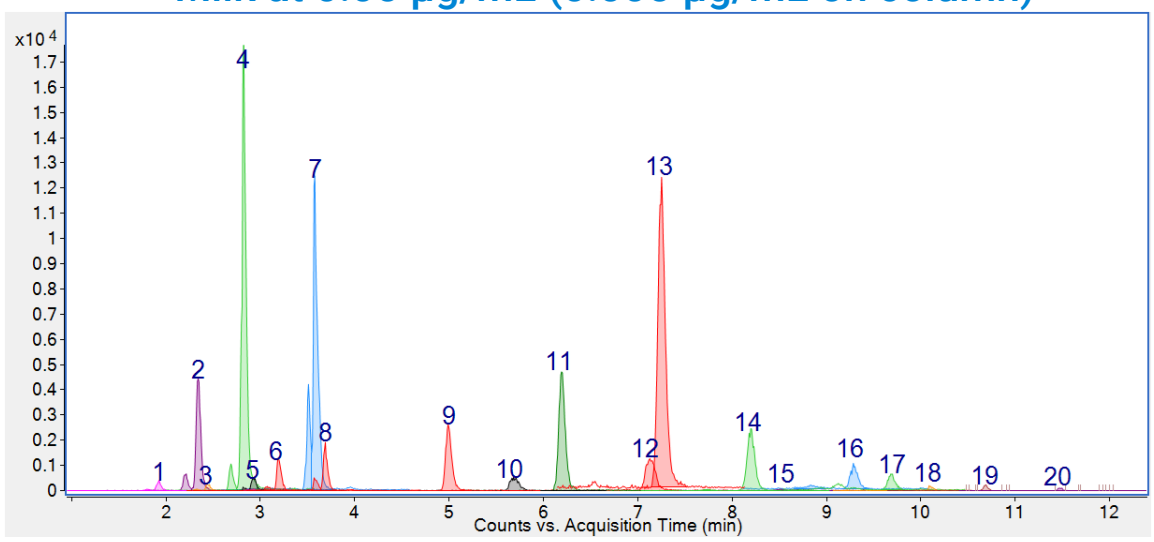
- ✓ A group of internal standards distributed across the retention time range and representatives of most lipid classes were selected to evaluate method performance.
- ✓ Three sets of standards (extracted matrix-matched standards (4 replicates), post-extraction matrix-matched standards and standards in solvent) were prepared at 0.01, 0.02, 0.05, 0.1, 0.5 and 1 µg/mL to test the linearity, limit of quantitation (LOQ), extraction efficiency, reproducibility, and matrix effect.

Results and Discussion

Method Validation Results

- ✓ Extracted matrix-matched standards provide accurate results by compensating for both matrix effects and potential recovery losses
- ✓ All the analyte corrected recoveries (CR, within the 88-130% range), precision (CV, ≤20%) and matrix effect (ME, 10-160%) were obtained at and above the LOQs
- ✓ The coefficient of determination (r^2) values of matrix extracted calibration curves were >0.99 for all the analytes ranging from 0.01 to 1 µg/mL

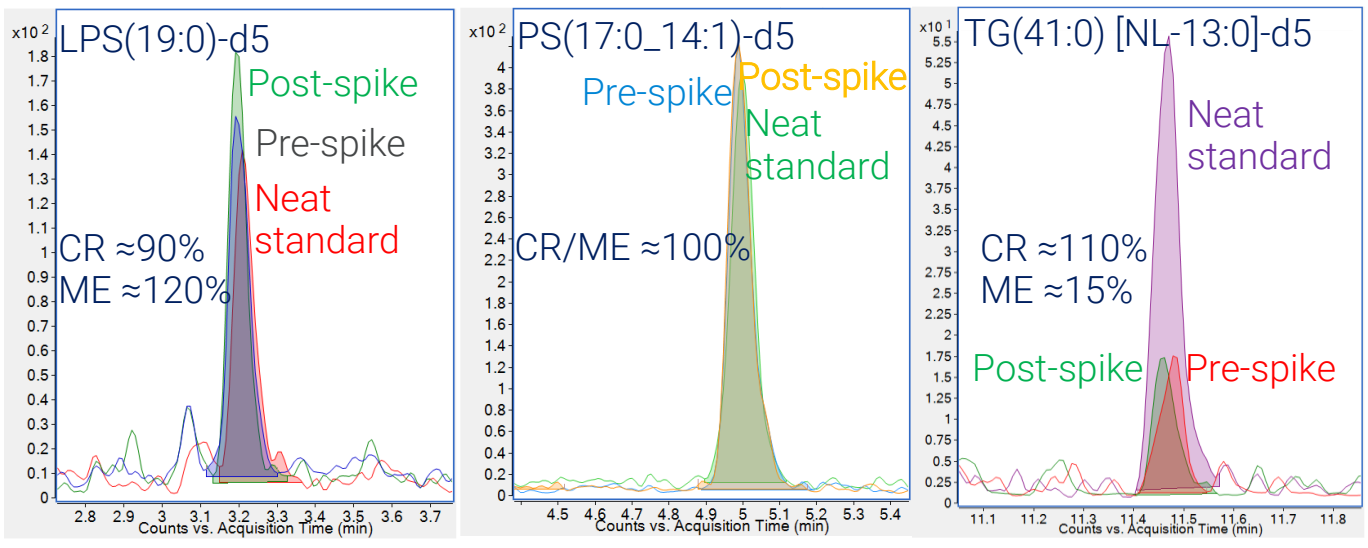
Elution Profile of Internal Standards Spiked in Human Milk at 0.05 µg/mL (0.005 µg/mL on column)



Method Validation Results

Internal Standard	No.	RT, min	Extracted matrix-match curve range µg/mL	Accuracy range for all calibration points on curve %	Coefficients of determination (R^2)	Corrected recovery range for all calibration points %	Precision range for all calibration points %	Matrix effect for all calibration points, %	LOQ in Human Milk µg/mL
LPS(15:0)-d5	1	1.92	0.01-1	85.3 - 126	0.9937	105 - 119	0.9 - 12.1	24 - 40	0.01
LPC(15:0)-d5	2	2.34	0.01-1	83.8 - 114	0.9944	117 - 130	0.8 - 11.3	27 - 46	0.01
LPE(15:0)-d5	3	2.43	0.01-1	82.7 - 124	0.9939	117 - 131	4.0 - 10.3	14 - 33	0.01
LPC(18:1)-d7	4	2.82	0.01-1	86.4 - 114	0.9955	116 - 122	1.8 - 11.8	79 - 111	0.01
LPE(18:1)-d7	5	2.92	0.01-1	83.6 - 127	0.9956	112 - 122	2.7 - 15.5	82 - 135	0.01
LPS(19:0)-d5	6	3.19	0.01-1	84.7 - 121	0.9939	98.2 - 105	1.5 - 11.1	108 - 142	0.01
LPC(19:0)-d5	7	3.58	0.01-1	82.1 - 129	0.9938	108 - 115	2.4 - 9.5	114 - 156	0.01
LPE(19:0)-d5	8	3.69	0.002-0.2	80.6 - 125	0.9938	113 - 120	3.6 - 9.7	80 - 109	0.002
PS(17:0_14:1)-d5	9	4.99	0.01-1	83.4 - 121	0.9928	96.3 - 109	1.4 - 11.7	82 - 109	0.01
PS(15:0_18:1)-d7	10	5.68	0.002-0.2	85.8 - 129	0.9933	93.4 - 104	1.5 - 12.5	87 - 141	0.002
PE(17:0_14:1)-d5	11	6.19	0.01-1	84.2 - 119	0.9946	114 - 122	2.7 - 12.0	94 - 124	0.01
PE(15:0_18:1)-d7	12	7.13	0.002-0.2	85.5 - 117	0.9953	117 - 124	2.6 - 13.1	85 - 112	0.002
SM(d18:1_20:1)-d9	13	7.25	0.01-1	87.7 - 114	0.9954	117 - 122	2.7 - 12.6	93 - 129	0.01
PE(17:0_22:4)-d5	14	8.20	0.01-1	77.4 - 119	0.9950	114 - 122	3.0 - 13.4	86 - 127	0.01
DG(17:0_14:1)-d5	15	8.50	0.02-1	72.8 - 129	0.9931	88.4 - 129	5.8 - 13.9	64 - 127	0.02
Cer(d18:1_20:1)-d7	16	9.30	0.01-1	86.1 - 119	0.9943	87.9 - 128	3.2 - 10.3	76 - 113	0.01
DG(15:0_18:1)-d7	17	9.62	0.04-1	74.4 - 124	0.9927	88.4 - 130	5.5 - 12.2	66 - 102	0.04
DG(17:0_22:4)-d5	18	10.10	0.01-1	84.9 - 118	0.9918	112 - 130	5.6 - 14.0	55 - 86	0.01
TG(41:0) [NL-13:0]-d5	19	10.69	0.01-1	81.2 - 125	0.9906	111 - 130	6.7 - 16.1	15 - 20	0.01
TG(57:4) [NL-21:2]-d5	20	11.50	0.01-1	72.8 - 126	0.9928	114 - 129	8.1 - 19.6	11 - 15	0.01

Examples of Corrected Recovery, Matrix Effect at 0.01 µg/mL in Sample



Conclusions

This targeted LC-MS/MS workflow provides a sensitive and analytically robust approach suitable for large-scale lipidomic characterization of complex biological matrices, including human milk, supporting ongoing studies of human milk composition and infant health.

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